The GPIIbIIIa antagonist drugs eptifibatide and tirofiban do not induce activation of apoptosis executioner caspase-3 in resting platelets but inhibit caspase-3 activation in platelets stimulated with thrombin or calcium ionophore A23187

Reagents and solutions

Integrilin (eptifibatide, 0.75 mg/mL) was purchased from Millennium Pharmaceuticals Inc. (Cambridge, MA) and Aggrastat (tirofiban hydrochloride, 50 μg/mL) was from Medicure Pharma Inc. (Somerset, NJ). Gly-Pro-Arg-Pro (GPRP) peptide was purchased from Sigma (St. Louis, MO), human α-thrombin was from Haematologic Technologies Inc (Crossing, VT), A23187 was from Calbiochem (San Diego, CA) and FAM-DEVD-FMK (carboxyfluorescein-carbonyl-aspartyl-glutamyl-valyl-aspartic acid fluoromethyl ketone) was from Chemicon International (Temecula, CA). Control diluent buffer A was composed of phosphate buffered saline (Invitrogen, Carlsbad, CA) supplemented with 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA and 10 mM HEPES, pH 7.4.

Treatment of platelets with GPIIbIIIa antagonists, thrombin and A23187, and determination of caspase-3 activation

In order to mimic an *in vivo* situation, in this study, we used platelet-rich plasma (PRP), rather than washed platelets, to investigate the effect of Integrilin and Aggrastat on caspase-3 activation in the presence of plasma proteins.

Venous blood from healthy volunteers was anticoagulated with 0.32% sodium citrate and PRP was obtained by centrifugation at 180 g for 15 min at room temperature (RT). Caspase-3 activation was determined as previously described[1,2] using the cell-penetrating carboxyfluorescein-labeled FAM-DEVD-FMK probe, which covalently binds to active caspase-3.[3] To study the effect of Integrilin and Aggrastat on caspase-3 activation in resting platelets, GPIIbIIIa antagonists at final concentrations of 0.48-4.8 μM or control diluent buffer A were incubated with citrated PRP for 45 min at RT. In platelets stimulated with thrombin or A23187, GPIIbIIIa antagonists at final concentration of 0.48 μM or buffer A were preincubated with PRP for 30 min at RT followed by incubation for 15 min at RT with either 1 U/mL human-α thrombin plus 2.5 mM GPRP or 10 μM A23187 (final concentrations).

For detecting caspase-3 activation, 5 μL aliquots of 10X working FAM-DEVD-FMK solution, prepared according to the manufacturer's recommendations, were added to 45 μL of treated platelet samples and incubated for 60 min at 37°C in the dark. Following dilution to 500 μL with buffer A, samples were acquired within 30-60 min, fluorescent (FL1) histograms were analyzed, and caspase-3 activation was quantified as the mean channel fluorescence (MCF) of platelet-bound FAM-DEVD-FMK.

Calculation of inhibitory effect of GPIIbIIIa antagonists on caspase-3 activation

To quantify the impact of Integrilin and Aggrastat on caspase-3 activation in platelets stimulated with thrombin and A23187, the percentage of inhibition of caspase-3 activation was calculated in each experiment by the formula:

Inhibition, % = (AG – AN) x 100% / (AG – CON), where AG: caspase-3 activation in platelets treated with platelet *ag*onist (thrombin or A23187), AN: caspase-3 activation in platelets treated with GPIIbIIIa *an*tagonist (Integrilin or Aggrastat) plus platelet agonist, and CON: caspase-3 activation in platelets treated with *con*trol diluent buffer A.

Statistical analysis

Data of seven experiments are presented as means ± SEM. The statistical significance of the differences between different platelet groups was determined by one-way ANOVA with Dunnett's multiple comparison *post hoc* test. Differences were considered significant when $p<0.05$.

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References

1. Leytin V, Allen DJ, Mykhaylov S, Lyubimov E, Freedman J. Thrombin-triggered platelet apoptosis. J Thromb Haemost 2006;4:2656-63.
2. Leytin V, Allen DJ, Mutlu A, Gyulkhandanyan AV, Mykhaylov S, Freedman J. Mitochondrial control of platelet apoptosis: Effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. Lab Invest 2009;89:374-84.
3. Smolewski P, Bedner E, Du L, Hsieh TC, Wu JM, Phelps DJ, Darzynkiewicz Z. Detection of caspases activation by fluorochrome-labeled inhibitors: Multiparameter analysis by laser scanning cytometry. Cytometry 2001;44:73-82.